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Optimization of 1, 3-Propanediol production by *Klebsiella pneumoniae* 141B using Taguchi methodology: Improvement in production by cofermentation studies

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In this study, 1,3-Propanediol production by isolated *Klebsiella pneumoniae* 141B strain using glycerol as a substrate has been investigated. Initial experiments were carried for the screening of nitrogen sources and yeast extract was found to be the optimum source. The impact of vitamin B12 on 1,3-PDO production was evaluated by growing the strain in vitamin B12 supplemented medium. One interesting phenomenon noticed that 1,3-PDO production was not increased by vitamin B12 supplementation, which aided in lowering the cost of fermentation process. Further optimization studies were carried out with six selected fermentation parameters such as pH, yeast extract, glycerol, calcium carbonate, inoculum size and temperature at three different levels. 1,3-PDO production has improved to 67% in Taguchi statistical optimization method. To further increase the 1,3-PDO production, cofermentation studies were carried out with the optimized medium having glucose and sucrose as co substrates. There was a sharp increase in the 1,3-PDO yield from control (0.70 mol/mol) having glycerol alone to glycerol-sucrose mixture (0.98 mol/mol) followed by glycerol-glucose mixture (0.84 mol/mol). The sucrose-glycerol mixture with vitamin B12-free medium allows the development of an economical biological process for the production of 1,3-PDO from renewable resources.

Key words: 1,3-propanediol, *Klebsiella pneumoniae*, glycerol, glucose, sucrose

1,3-Propanediol (1,3-PDO or trimetylene glycol) has traditionally been considered as a 'specialty chemical' is now undergoing a transition into a 'commodity chemical' (Sheldon *et al.,* 2007). It has been gaining great importance recently due to its role as a monomer in the synthesis of polyesters for fabric and textile applications. 1,3-PDO also finds application in making transparent ballistic polymer which is a life-saving technology, and the war fighter is the primary beneficiary. Ballistic polymer is based on a family of transparent materials whose composition can be tailored to enhance properties such as transparency, impact resistance, and UV stability (Saxena *et al.,* 2009).

The use of natural organisms to produce 1,3-propanediol is well studied in bacteria. The natural producers of 1,3-propanediol from glycerol are of genera *Klebsiella, Clostridia, Citrobacter* and *Lactobacilli* (Sauer *et al.,* 2008). Among these organisms, *C. butyricum* and *K. pneumoniae,* are considered the best "natural producers" and are paid more attention because of their

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appreciable substrate tolerance, yield, and productivity (Gonzalez-Pajuelo *et al.,* 2006). In the metabolic reactions, glycerol is dissimilated through coupled oxidative and reductive pathways (Yang *et al.,* 2007). The reductive pathway is carried out in two enzymatic steps. The first enzyme vitamin B12-dependent glycerol dehydratase (GDHt) removes a water molecule from glycerol to form 3-hydroxypropionaldehyde (3-HPA), which is then reduced to 1,3-PDO by second enzyme, NADH linked 1,3-propanediol oxidoreductase (PDOR).

In the present study, we have isolated a new strain of *Klebsiella pneumoniae* 141B for 1,3-PDO production. For efficient microbial 1,3-PDO production, fermentation parameters were optimized by Taguchi design of experimental methodology. In our previous work, we have reported the production of 3-HPA, an intermediate product in the production of 1,3-PDO using glucose as a substrate by a novel strain *Enterococcus dispar* (Vanajakshi and Annapurna, 2010). In this study, an attempt has also made to evaluate the impact of different co substrates such as glucose and sucrose on 1,3-PDO production by *K. pneumonia* strain.

MATERIALS AND METHODS

Microorganism

In present study, the organism used for the production of 1,3-PDO was an isolated strain from soil samples collected from Indian Institute of Chemical Technology, Hyderabad. The strain was identified as *Klebsiella pneumoniae* based on morphological, physiological and biochemical characteristics. It was deposited in IMTECH, Chandigarh with the accession number of *K. pneumoniae* MTCC 9751. The 16S rDNA sequence of this strain displayed the highest degree of homology (98%) with the *Klebsiella* sp. Its EMBL accession number was FN820293.

Medium and culture conditions

The strain was maintained on agar slant medium containing (g/L) yeast extract, 5; peptone, 10; NaCl, 9; glycerol, 20; and agar, 20. For seed culture development, the strain was grown on medium consisting of all above components except agar. The production medium used for 1,3-PDO production contained (g/L) glycerol, 20; K_2HPO_4, 0.69; KH_2PO_4, 0.25; $(NH_4)_2SO_4$, 6; $MgSO_4.7H_2O$, 0.2; yeast extract, 1.5; and 1 ml of trace element solution. The composition of the trace element solution was (mg/L): $MnSO_4.4H_2O$, 100; ZnCl2, 70; $Na_2MoO_4.2H_2O$, 35; H_3BO_3, 60; $CoCl_2.6H_2O$, 200; $CuSO_4.5H_2O$, 29.28; NiCl2.6H2O, 25; $FeSO_4$ solution, 0.2 ml and 37% HCl, 0.9 ml. The experiments were carried out by batch fermentation in 250 ml Erlenmeyer flasks containing 100 ml of production medium with 2% (v/v) seed culture. After inoculation, the flasks were incubated in an orbital shaker (Labtech, Germany) at 37°C, 150 rpm for 8 h and analyzed for 1,3-PDO production. All experimental trials were performed in triplicate.

Optimization of process parameters

Various process parameters influencing 1,3-PDO production during submerged fermentation were optimized. In order to investigate the influence of nitrogen sources, the medium was supplemented with different organic nitrogen sources such as urea, peptone, beef extract, yeast extract, malt extract, soybean meal, casein, tryptone and gelatin, and inorganic nitrogen sources such as ammonium chloride, ammonium sulphate, ammonium nitrate, potassium nitrate, and sodium nitrate. The respective nitrogen sources were added as a sole source of nitrogen (7.5 g/L) to the production medium. The effect of substrate concentration on 1,3-PDO production was studied by varying the glycerol concentrations from 20-100 g/L. Glycerol dehydratase (GDHt), a key enzyme involved in the production of 1,3-PDO

requires vitamin B12 as a coenzyme (Sauer *et al.*, 2008). It catalyzes the conversion of glycerol to 3-HPA, an intermediate compound in the 1,3-PDO metabolic pathway. To study the impact of vitamin B12 on 1,3-PDO production, the isolate was grown in a medium supplemented with varying vitamin B12 concentrations ranging from 0.0025-0.01 g/L. A control flask was maintained without adding vitamin B12.

Statistical optimization and analysis of the results

The optimum concentrations of production medium components for 1,3-PDO production from glycerol by *K. pneumoniae* were determined statistically by means of Taguchi design of experimental (DOE) methodology. DOE is an experimental strategy in which effects of multiple factors are studied simultaneously by running tests at various levels of the factors. L-18 orthogonal array of Taguchi experimental design was used for optimization and Qualitek-4 software (Nutek Inc., Bloomfield Hills, CA) was used for analyzing the results. Six different fermentation parameters such as pH, yeast extract, glycerol, calcium carbonate, inoculum and temperature were selected at three different levels.

Cofermentation studies between glycerol and sugars

Cofermentation of glycerol with sugars was critical for complete bioconversion of glycerol to 1,3-PDO. Glucose and sucrose were the sugars chosen for cofermentation studies. A set of batch fermentations were carried out with sugars and glycerol at a molar ratio of 0.1, 0.2, 0.3, 0.4 and 0.5 mol/mol respectively by *K. pneumoniae* under aerobic conditions. A control flask was maintained where glycerol fermentation takes place alone without adding any sugar. Samples were taken periodically throughout the course of the fermentations and analyzed for 1,3-PDO, sugar and byproduct concentrations.

Analytical methods

The concentration of glycerol, glucose, 1,3-PDO, 2,3-butanediol, succinate, acetic acid and ethanol were determined by a high-performance liquid chromatography system (SHIMADZU 10A) equipped with an Aminex HPX-87H column (Bio-Rad) with a refractive index detector. The working conditions were: 0.005M H_2SO_4 as a mobile phase with a flow rate of 0.5ml/min, and 65^oC as the working temperature. Biomass was determined by dry cell weight (DCW) method (Herbert *et al.*, 1971). GDHt activity was determined by the modified 3-methyl-2-benzothiazolinone hydrazone (MBTH) method (Toraya *et al.*, 1980) and PDOR enzyme was assayed according to the method described by Johnson and Lin (Johnson and Lin, 1987).

RESULTS AND DISCUSSION

In this study, 1,3-PDO production was carried out from glycerol at an initial concentration of 20 g/L by isolated *K. pneumonia*e 141B strain. The fermentation profile of 1,3-PDO production was shown in Fig. 1. It could be observed from the figure that the total glycerol supplied in the production medium (20 g/L) was consumed with in 8 h and the maximum 1,3-PDO production of 10.18 g/L was recorded at the same fermentation time. Maximum biomass of 3.12 g/L was noticed at 5th h of fermentation time, where the active metabolite production was initiated. Ethanol, 2,3-butanediol and succinic acid were the major byproducts produced by this strain. Among these byproducts ethanol was the principle byproduct produced at a concentration of 1.48 g/L followed by 2,3-butanediol and succinic acid at 1.08 and 0.81 g/L respectively. In general, 1,3-PDO was produced from the reductive pathway and by products were produced from the oxidative

pathway of glycerol metabolism (Gungormusler *et al.,* 2010). Production of both 1,3-PDO and byproducts were decreased beyond the optimum fermentation time of 8 h, this might be due to the depletion of nutrients or accumulation of secondary metabolites, which resulted in the inhibition of the enzymes involved in the production of 1,3-PDO and byproducts.

Evaluation of nitrogen sources for 1,3-PDO production

Influence of nitrogen sources on 1,3-PDO production was investigated, at a concentration of 7.5 g/L. The order of production of 1,3-PDO under the influence of various nitrogen sources was yeast extract > beef extract > tryptone > ammonium chloride > peptone > ammonium sulphate > malt extract > potassium nitrate > urea > ammonium nitrate >soyabean meal > sodium nitrate (Fig. 2). Results revealed that among selected nitrogen sources, yeast extract had shown the maximum 1,3-PDO production of 11.54 g/L followed by beef extract at 9.89 g/L. Further evaluation of yeast extraction concentration showed a parabolic nature of production pattern (Fig. 2 insert). There was an increase in 1,3-PDO production with an increase in the concentration of yeast extract up to 7.5 g/L and thereafter, a decline in this function was recorded. The yield of 1,3-PDO obtained at 7.5 g/L yeast extract was found to be 11.56 g/L. The higher concentration of yeast extract was inhibitory for 1,3-PDO synthesis. This could be due to the feedback inhibition of yeast extract on the enzymes involved in the 1,3-PDO synthesis. In this study, yeast extract was found to be the optimum nitrogen source for 1,3-PDO production by this strain. Such yeast extract dependent variation of 1,3-PDO production was also reported with other microbial strains (Chen *et al.,* 2005; Zhang *et al.,* 2006). Among inorganic nitrogen sources tested, ammonium chloride had shown the maximum 1,3-PDO production of 8.47 g/L followed by ammonium sulfate, which resulted in 7.68 g/L of 1,3-PDO (Fig. 2). Zhang *et al*. (2007) found the ammonium sulfate as the optimum nitrogen source for 1,3-PDO production with an yield of 8.09 g/L by *K. pneumoniae* XJ-Li strain. Complex organic nitrogen sources were more suitable for 1,3-PDO production than inorganic nitrogen sources as they act as both energy and nitrogen sources for the organism.

Influence of glycerol concentration on 1,3-PDO production

To determine the optimum substrate concentration for 1,3-PDO production, glycerol concentration was varied from 20 - 100 g/L. As the glycerol concentration increased from 20-90 g/L, 1,3-PDO production also increased from 11.61 to 41.35 g/L (Fig. 3). Further increase in glycerol concentration to 100 g/L, resulted in the declined production of 38.79 g/L. Though maximum production was achieved at 90 g/L, the conversion efficiency of glycerol to 1,3-PDO was high at initial glycerol concentrations of 20 and 30 g/L with a molar yield of 0.702 and 0.706 respectively, when compared to low molar yield of 0.46 mol/mol at 90 g/L glycerol. This might be due to the rapid suicidal inactivation of glycerol dehydratase (GDHt) at high glycerol concentrations (Toraya, 2003). In the present study, high productivity of 1.82 g/L/h was achieved by the present strain at 40 g/L glycerol concentration. Productivity of *K. pneumoniae* strain was increased from 20 to 40 g/L, and it was decreased at high glycerol concentrations. The productivity reported by this strain at 40 g/L glycerol was 1.82 g/L/h, which was remarkably higher than the previous reports who reported 0.19 g/l/h at the same concentration (Zhang *et al*., 2007).

The fermentation time required for this strain to convert 20 g/L glycerol to 1,3-PDO was 8 h with a molar yield of 0.70 mol/mol glycerol. Earlier studies with *K. pneumoniae*, *Citrobacter freundii*, *Enterobacter*

agglomerans and *Clostridium butyricum* resulted in low molar yields of 0.65, 0.65, 0.51 and 0.62 with the fermentation times of 11, 12, 14 and 9 h respectively at 20 g/L glycerol concentration (Barbirato *et al*., 1998). Hao *et al*. (2008) reported 13.7 g/L of 1,3-PDO with a molar yield of 0.55 in 11 h at 30 g/L glycerol concentration, where as the present strain yielded 17.52 g/L of 1,3-PDO with a molar yield of 0.70 in 10 h at the same glycerol concentration. The above results indicate that isolated *K. pneumoniae* strain was a potent strain capable of 1,3-PDO production. Biomass concentration of *K. pneumoniae* strain was increased from 2.8 to 5.4 g/L with the increase in glycerol concentration from 20 to 90 g/L. 2,3-butanediol, a value added byproduct of 1,3-PDO fermentation, was also increased with the increase in glycerol concentration from 20 to 60 g/L and maximum production of 2.81 g/L was observed at 60 g/L glycerol concentration. Ethanol and succinic acid were produced at maximum concentration of 5.82 and 3.98 g/L respectively at 70 g/L glycerol concentration.

Impact of vitamin B12 on 1,3-PDO production

All the natural 1,3-PDO producers such as *K. pneumoniae*, *Citrobacter freundii* and *Clostridium pasteurianum* requires vitamin B12 as a coenzyme for GDHt enzyme during the conversion of glycerol to 1,3-PDO. In this study, influence of vitamin B12 concentration on the production of 1,3-PDO, GDHt and PDOR was investigated by varying its concentration from 0.0025 to 0.01 g/L and a control was maintained without adding vitamin B12. Interestingly, control had shown the maximum production of 1,3-PDO, GDHt and PDOR enzymes when compared to vitamin B12 supplemented media (Fig. 4). This could be due to the organism might have the ability to synthesize vitamin B12 naturally or the GDHt of this strain might be a coenzyme B12 independent enzyme. Saint-Amans *et al.* (2001) reported that the GDHt of *Clostridium butyricum* VPI1718, extracted from 1,3-PDO producing cells, was not stimulated by coenzyme B12, and they suggested it as a coenzyme B12-independent enzyme. Celine *et al.* (2003) reported on the cloning, sequencing, and characterization of the genes encoding the GDHt of *C. butyricum* VPI 1718.

Maximum production of GDHt and PDOR were observed in control at 1.14 and 1.41 U/mg respectively, and hence more 1,3-PDO production of 11.5 g/L was noticed in control (Fig. 4). When compared to control, there was not much change in the production of GDHt, PDOR and 1,3-PDO at lower vitamin B12 concentration (0.0025-0.0075 g/L), but at higher concentration (0.01 g/L), there was a decrease in the production of 1,3-PDO, GDHt and PDOR to 8.35 g/L, 0.87 and 0.66 U/mg respectively. The maximum GDHt production obtained with the present strain was 1.14 U/mg, which was in line with the earlier reports of Barbirato *et al*. (1996), who reported maximum level of glycerol dehydratase activity at 1.0 U/mg. The production of PDOR enzyme was 1.26 U/mg, which was higher than the previous reports of Menzel *et al*. (1997a). In general, most of the 1,3-PDO producing organisms requires the addition of high cost vitamin B12 molecule to the production medium. In this study, additional supplementation of vitamin B12 was not required for the isolated *K. pneumoniae* strain, which allows the development of an economical vitamin B12-free biological process for the production of 1,3-PDO.

Statistical optimization of 1,3-PDO production

Preliminary investigations revealed that glycerol, yeast extract, calcium carbonate, inoculum size, pH and temperature were the critical medium components for 1,3-PDO production by this isolated strain. In view of the isolated strain's higher 1,3-PDO production potential, further interaction behaviour of culture conditions which play

vital role in economizing the fermentation process was investigated using L-18 orthogonal array of Taguchi methodology. The above six fermentation factors were taken at three different levels for further evaluation of 1,3-PDO production (Table 1). Experimental plan layout, along with 1,3-PDO production values was depicted in Table 2.

Table 1. Selected fermentation factors for the production of 1,3-PDO by *K. pneumoniae*

S. No	Factor	Level 1	Level 2	Level 3
1	Unused	-	-	-
2	pH	6	7	8
3	Yeast extract (g/L)	5.0	7.5	10
4	Glycerol (g/L)	20	40	60
5	$CaCO_3$ (g/L)	2.5	5.0	7.5
6	Inoculum (% v/v)	1	2	3
7	Temperature (^oC)	34	37	40
8	Unused	0	0	0

Table 2. L18 orthogonal experimental array for the production of 1,3-PDO by *K. pneumoniae*

S. NO	Factors								1,3-PDO (g/L)
	1	2	3	4	5	6	7	8	
1	0	1	1	1	1	1	1	0	9.60
2	0	1	2	2	2	2	2	0	24.4
3	0	1	3	3	3	3	3	0	25.8
4	0	2	1	1	2	2	3	0	7.40
5	0	2	2	2	3	3	1	0	24.1
6	0	2	3	3	1	1	2	0	25.4
7	0	3	1	2	1	3	2	0	18.8
8	0	3	2	3	2	1	3	0	17.5
9	0	3	3	1	3	2	1	0	8.20
10	0	1	1	3	3	2	2	0	32.2
11	0	1	2	1	1	3	3	0	7.40
12	0	1	3	2	2	1	1	0	18.5
13	0	2	1	2	3	1	3	0	15.4
14	0	2	2	3	1	2	1	0	28.9
15	0	2	3	1	2	3	2	0	12.5
16	0	3	1	3	2	3	1	0	26.4
17	0	3	2	1	3	1	2	0	9.80
18	0	3	3	2	1	2	3	0	9.40

Trend of influence of factors on 1,3-PDO production

By studying the main effects of each of the factors, the general trend of the influence of the factors toward the process can be characterized. Evaluation of data based on L-18 orthogonal array (OA) experimental design indicated a variation of 1,3-PDO production values from 7.4 to 32.2 g/L, suggesting the imperative role of selected factors and their concentration on product yield. Factor analysis at individual level revealed that yeast extract and temperature at level 2, pH at level 1 and glycerol, calcium carbonate and inoculum size at level 3 were optimum for 1,3-PDO production (Fig. 5). Among all the selected factors, glycerol was the most influential factor as the change in its concentration from 20 to 60 g/L contributed maximum variability of 26.03 g/L. Temperature was the next factor that exhibited significant contribution to variability at 20.5 g/L, followed by pH at 19.6 g/L, calcium carbonate at 19.2 g/L, inoculum size at 19.1 g/L and least influence was exhibited by yeast extract concentration at 18.6 g/L. In the present study, glycerol concentration had a detrimental role on 1,3-PDO production, such carbon source concentration dependent 1,3-PDO production regulation has been noticed in different microbial strains (Barbirato *et al.,* 1998).

Interaction studies

Understanding the impact of each individual factor is the key for a successful fermentation process. The interaction effect between two factors was measured in terms of a numerical quantity known as interaction severity index (SI) value, calculated by Qualitek-4 software program and expressed in terms of %. From Table 3, it could be inferred that strength of interaction between pH and calcium carbonate was the highest (73.53%) followed by pH and temperature (64.49%). Interaction between yeast extract and calcium carbonate elicited least SI value (0.52%). It is interesting

to observe that calcium carbonate was the same factor involved in the highest and least SI values indicating that fermentation factors were interacting differentially in the fermentation process. Though glycerol influenced 1,3-PDO production greatly at individual level (Fig. 5), its interaction with other factors did not show significant effect on the 1,3-PDO yield (Table 3). Yeast extract at individual level (Fig. 5) showed the least influence on 1,3-PDO production, but its interaction with other factors such as pH and temperature elucidated a remarkable SI values of 56.77 and 55.96% respectively and moreover these two factors were physical factors (Table 3). pH interaction was noticed to be highest among all studied factors (Table 3) suggesting its importance in 1,3-PDO production by this strain, even though it was the third important factor at individual level. This could be evidenced based on the observation that it showed maximum interaction with temperature (64.49%), yeast extract (56.77%) and inoculum (42.3%) in addition to calcium carbonate (73.53%). However, in the present experiment a very less interaction of 7.68% was noticed between pH and glycerol, a high influencing factor at individual level in 1,3-PDO fermentation process.

ANOVA (Analysis of Variance)

ANOVA was used to analyze the results of the OA experiment and to determine how much variation each factor has contributed. F-ratios (in ANOVA), reveals the significance of the controlling factors for the fermentation process. It is inferred from F-ratios that glycerol was the most significant factor (74.2%) followed by temperature (13.14%) in the overall 1,3-PDO production by this strain (Table 3). More than 74% contribution in 1,3-PDO production was observed with glycerol alone and nearly 20% contribution noticed with physical parameters such as pH and temperature. Of all the selected factors yeast extract (1.16%) was found to be the least significant at individual level.

Optimum conditions and validation

Optimum conditions and individual parameter performance in terms of contribution for achieving higher 1,3-PDO yield were shown in Table 3. Based on this information, optimum 1,3-PDO yields could be achieved at pH 6 with 7.5 g/L of yeast extract, 60 g/L glycerol, 7.5 g/L calcium carbonate using 3% v/v inoculum at a fermentation temperature of 37^{o}C. These optimum conditions revealed the importance of physical factors and media components along with their concentrations in 1,3-PDO production with this microbial strain. The average performance of this strain in 1,3-PDO production was observed to be 17.87 g/L which represent > 52% in overall production (Table 3). However, all fermentation factors contribution was noticed to be 16.06 g/L, which represents > 47% in overall production. The total 1,3-PDO production under optimized conditions was predicted to be 33.93 g/L by the statistical procedure (Table 3). Validation experimentation using software predicted optimum conditions revealed maximum 1,3-PDO production of 33.22 g/L by this *K. pneumonia strain*, which proves the validity of Taguchi approach.

Cofermentation studies with glucose and sucrose as co substrates

The ratio between two substrates is an important parameter in microbial fermentations to predict the optimum combination for achieving higher yields. In this work, glucose and sucrose were evaluated as co substrates in glycerol fermentation at a concentration of 0.1-0.5 mol/mol of glycerol. The results revealed that there was a significant lag phase in glycerol utilization during the first 4 h where glucose was metabolized initially by this strain. From 5th onwards both glycerol and glucose were metabolized simultaneously

and glucose was completely consumed in 7 h (data not shown). Therefore, glucose alone was used during exponential growth followed by simultaneous consumption of glucose-glycerol has been noticed. Similar observation has been noted by Abbad-Andaloussi *et al*. (1998), while working with *Clostridium butyricum* DSM 5431 strain. On the contrary, aerobic growth of *Lactobacillus rhamnosus* on glycerol/glucose media resulted in onset of glycerol uptake only after complete exhaustion of glucose from the growth medium (Alvarez *et al*., 2004). The fermentation time was increased from 8 to 9.5 h, in glycerol fermentation to glycerol-glucose cofermentation respectively.

Table 3. Interaction influence, ANOVA and optimized conditions for selected factors during 1,3-PDO production by *K. pneumonia*

Factors	pH	Yeast extract	Glycerol	$CaCO_3$	Inoculum	Temp	Other /error	Total
Interaction (severity index in %)								
pH	NA	56.77	7.68	73.53	42.3	64.49	-	244.77 (48.95)*
Yeast extract	56.77	NA	16.94	0.52	15.96	55.96	-	146.15 (29.23)
Glycerol	7.68	16.94	NA	14.39	4.06	4.55	-	47.62 (9.524)
$CaCO_3$	73.53	0.52	14.39	NA	15.18	24.37	-	127.99 (25.598)
Inoculum	42.3	15.96	4.06	15.18	NA	15.57	-	93.07 (18.614)
Temperature	64.49	55.96	4.55	24.37	15.57	NA	-	164.94 (32.988)
ANOVA								
DOF	2	2	2	2	2	2	5	17
Sum of Squrs	74.858	14.254	857.974	21.405	32.121	152.591	1.95	1155.156
Variance	37.429	7.127	428.987	10.702	16.06	76.295	0.39	-
F-ratio	95.944	18.27	1099.657	27.435	41.169	195.575	-	
Pure sum	74.077	13.474	857.193	20.625	31.34	151.811	-	
Percentage	6.412	1.166	74.205	1.785	2.713	13.142	0.577	100 %
Optimum conditions								
Level desc	6	7.5	60	7.5	3	37	-	
Level	1	2	3	3	3	2	-	
Contribution	1.777	0.811	8.161	1.377	1.294	2.644	-	

Total contribution from all factors-16.064 g/L

Current grand average of performance-17.872 g/L

Expected result at optimum condition-33.936 g/L

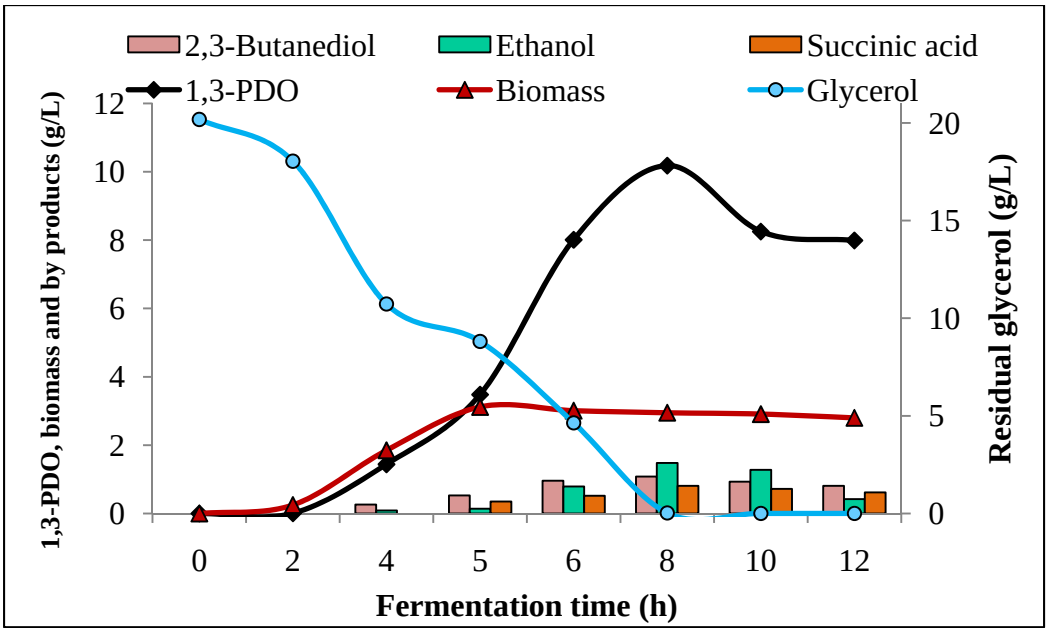

Fig. 1. Profile of 1,3-PDO production from glycerol by *K. pneumoniae*

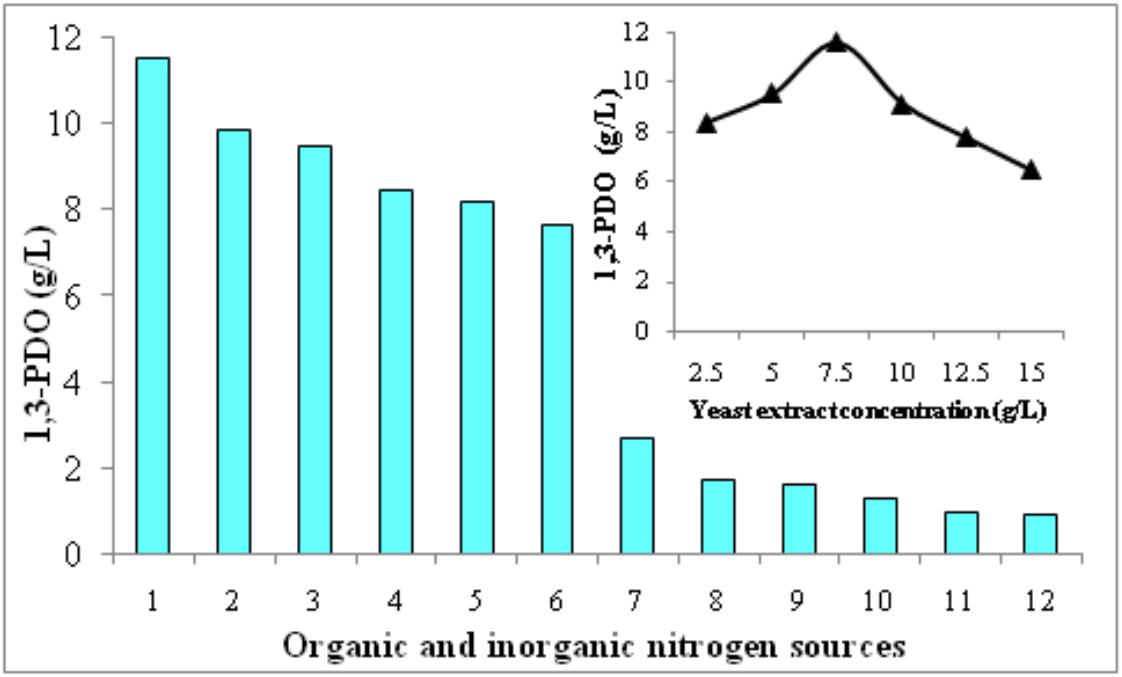

Fig. 2. Screening of different nitrogen sources for 1,3-PDO production by *K. pneumoniae*. (1) yeast extract; (2) beef extract; (3) tryptone; (4) NH_4Cl; (5) peptone; (6) $(NH_4)_2SO_4$; (7) malt extract; (8) KNO_3; (9) urea; (10) NH_4NO_3; (11) soya bean meal; (12) $NaNO_3$.

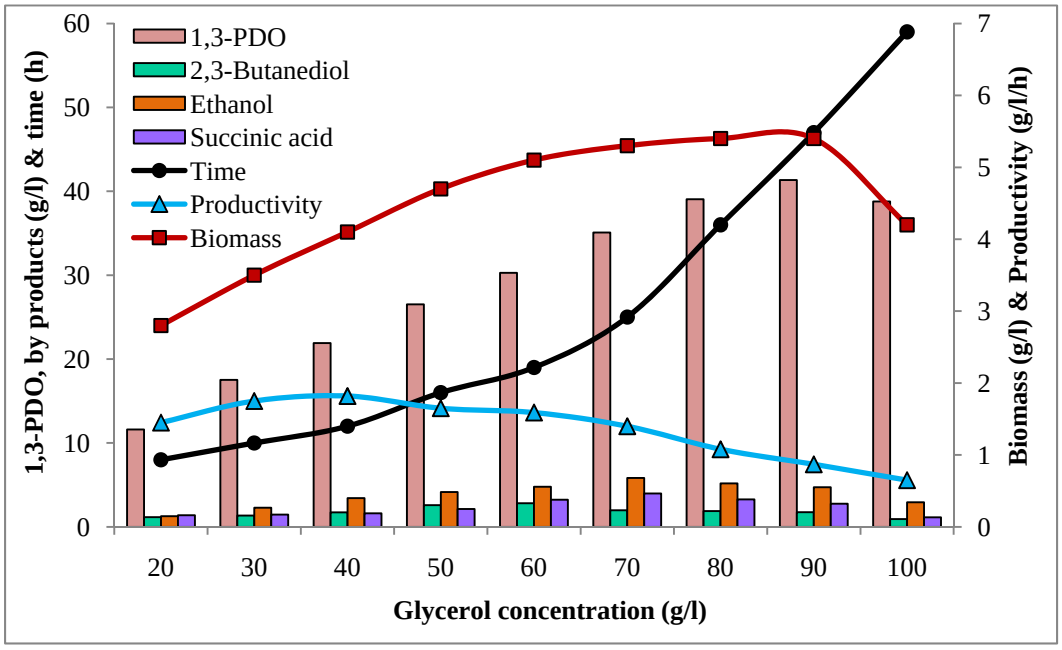

Fig. 3. Effect of glycerol concentration on 1,3-PDO production

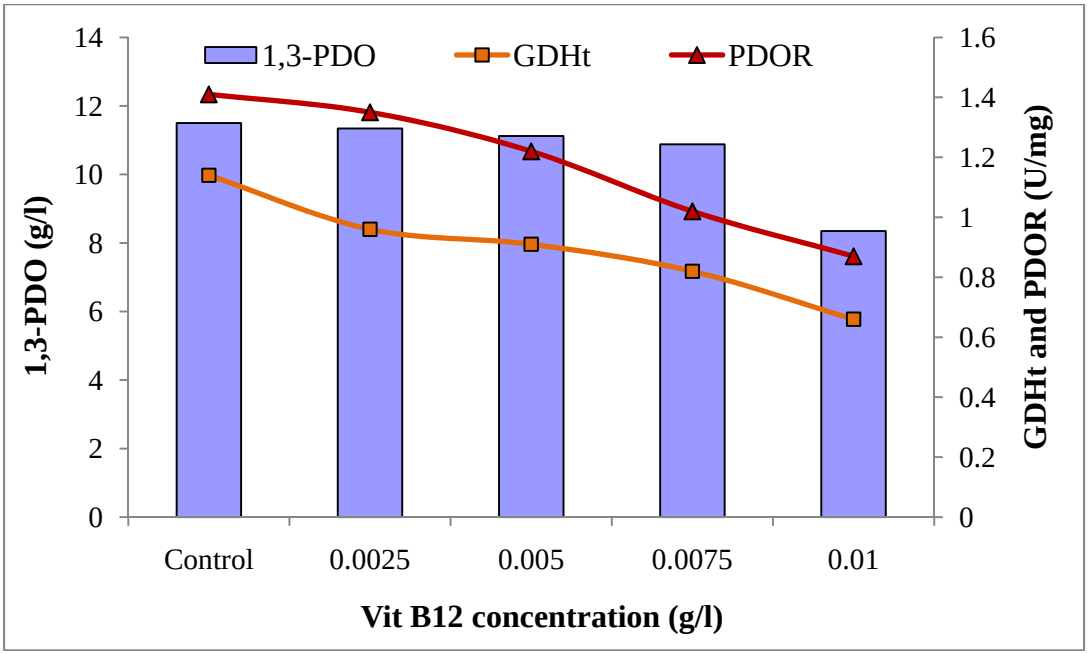

Fig. 4. Impact of vitamin B12 concentration on the production of 1,3-PDO, GDHt and PDOR by *K. pneumoniae*.

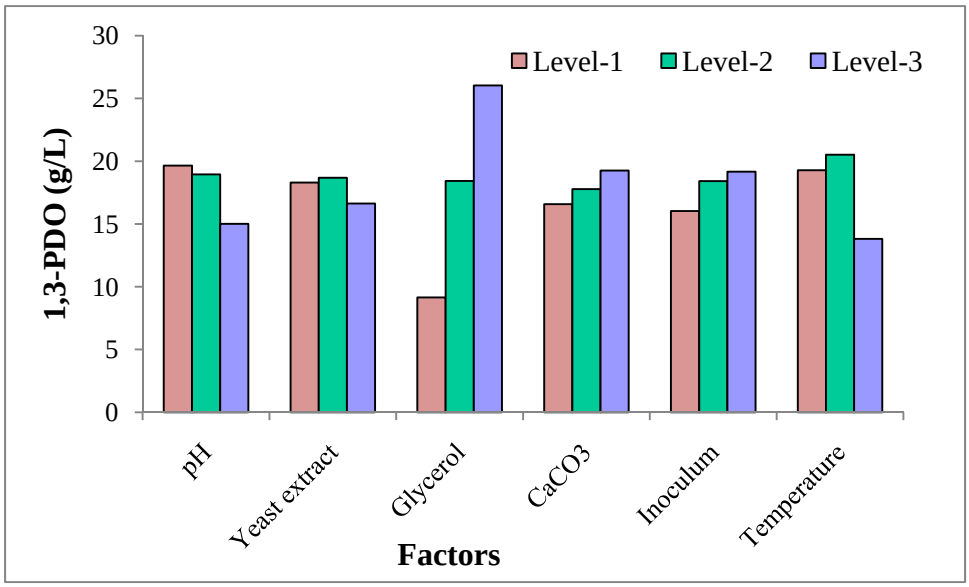

Fig. 5. 1,3-PDO production at selected fermentation parameter levels by *K. pneumoniae*.

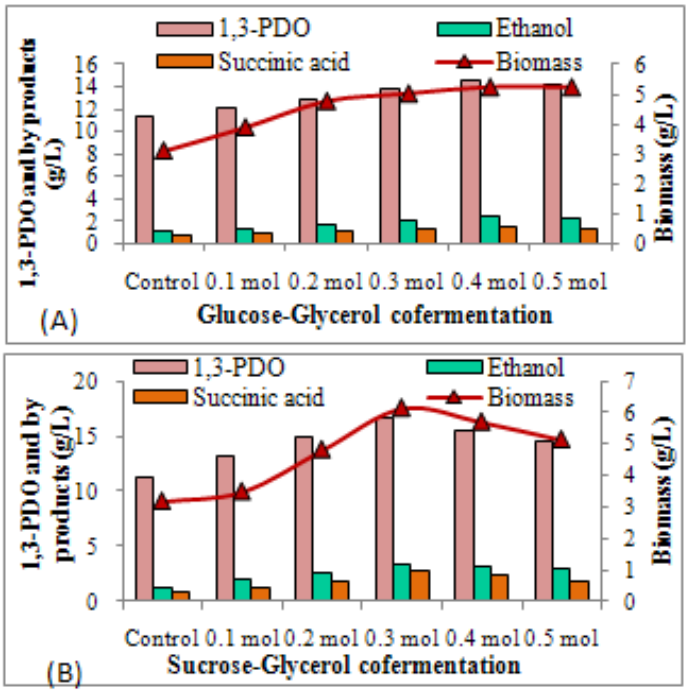

Fig. 6. 1,3-PDO production by *K. pneumoniae* with various co substrates. (A): Glucose as a co substrate, (B): Sucrose as a co substrate.

Experimental results of batch culture by *K. pneumonia* under aerobic conditions with glucose as a co substrate were shown in the Fig. 6 (A). There was a sharp increase in the 1,3-PDO yield from control having glycerol alone to glycerol-glucose mixtures. 1,3-PDO yield has been progressively increased from 0.1 to 0.4 mol/mol glucose-glycerol concentration and at 0.5 mol/mol glucose-glycerol ratio, the yield has been reduced, which could be due to inhibition of GDHt and PDOR enzymes by high glucose concentration. Xiu *et al*. (2007) revealed the glycerol-glucose theoretical ratio required for complete conversion of glycerol to 1,3-PDO was 0.32 mol/mol, which was close to the actual obtained experimental value at 0.4 mol/mol. In this study, 57% of glycerol was utilized for 1,3-PDO formation by reductive pathway and the remaining 43% was diverted for biomass and by product formation by oxidative pathway. The yield of 1,3-PDO has been increased from 0.70 mol/mol in glycerol alone fermentation to 0.84 mol/mol in glycerol-glucose cofermentation at a molar ratio of 0.4 mol glucose per mol glycerol. The reason could be attributed to the utilization of glucose catabolism for the generation of energy and glycerol was used solely for 1,3-PDO production resulting in the enhanced yield. In glycerol-glucose cofermentation, glucose, is often used as a H-donor substrate instead of the fraction of glycerol to provide both reducing equivalents for 1,3-PDO formation and ATP for biomass (Tran-Dinh *et al*., 1987). And moreover, the excess NADH liberated during glucose metabolism will be oxidized in the glycerol metabolism to increase the 1,3-PDO yield as the NADH produced during glycerol metabolism was not sufficient for 1,3-PDO formation (Abbad-Andaloussi *et al*., 1996).

The 1,3-PDO yield obtained by the present strain during glycerol-glucose cofermentation studies was 0.84 mol/mol of glycerol, which was higher than previous reports, who reported 0.62 mol/mol with the same *Klebsiella* sps (Xiu *et al*., 2007), and 0.60 mol/mol glycerol by *Clostridium butyricum* VPI 3266 strain (Saint-Amans and Soucaille, 1995). But in comparison to Biebl and Marten (Biebl and Marten, 1995), the yield obtained by this strain was less as they showed 90% glycerol conversion with mixed substrates by *Clostridia* sp. The reason could be due to formation of more byproducts by this *K. pneumoniae* strain and moreover the theoretical conversion rate suggested for *Clostridia* sps was 72%, which was higher than the 64% for *Klebsiella* sp, hence the yield might have increased in *Clostridia* sp. In the present study, the volumetric productivity of 1,3-PDO has been increased from control to glucose-glycerol mixture at 0.4 mol/mol in the range of 1.40 to 1.54 g/L/h respectively, even though the fermentation time has increased from 8 h to 9.5 h. Because of addition of glucose, biomass concentration was increased from control (3.11 g/L) to glycerol-glucose cofermentation (5.23 g/L) which was reflected in terms of enhanced formation of by products such as ethanol and succinic acid at 2.42 and 1.57 g/L respectively.

Experimental results of batch culture by *K. pneumonia* with sucrose as a co substrate at different molar concentrations (0.1 to 0.5 mol/mol of glycerol) were shown in the Fig. 6 (B). Among sucrose and glucose, sucrose had resulted in the maximum 1,3-PDO titer of 0.98 mol/mol glycerol at 0.3 mol/mol of sucrose-glycerol concentration. The reason for enhanced 1,3-PDO production could be that the organism might have an invertase enzyme capable of cleaving sucrose into glucose and fructose, thereby providing the plenty of energy source for organisms growth, hence the strain might diverting the total glycerol supplemented in the fermentation medium for 1,3-PDO production. The yield obtained by isolated *K.*

pneumoniae strain with sucrose as a co substrate was higher than literature cited yields such as, Yang *et al.* (2007) reported 1,3-PDO yield of 0.62 mol/mol glycerol with *Klebsiella oxytoca* M5a1 with sucrose as a cosubstrate. Malaoui and Marczak, (2001) reported 1,3-PDO yield of 0.90 mol/mol while working with *Clostridium butyricum* E5-MD. In this study, ethanol and succinic acid were also produced in high concentration at 3.35 and 2.83 g/L with 0.3 mol/mol of sucrose-glycerol cofermentation and maximum biomass of 6.13 g/L was observed at the same molar concentration. Hence sucrose is recommended as a co substrate due to its cost effectiveness to make the 1,3-PDO production as economically feasible process.

Conclusion

Over the past few decades, there has been growing interest in 1,3-PDO as an industrial chemical. In this study, 1,3-PDO production was carried out from glycerol by an isolated *K. pneumoniae* 141B strain. The impact of different fermentation parameters on 1,3-PDO production by an isolated *K. pneumoniae* strain was investigated using Taguchi methodology. Among all selected factors, glycerol was the most influential factor with the F-ratio of > 74% followed by temperature at 13%. The optimum condition for 1,3-PDO production by this strain were: pH-6, yeast extract-7.5 g/L, glycerol-60 g/L, calcium carbonate-7.5 g/L, inoculum size-3% (v/v) and fermentation temperature-37^{o}C. Cofermentation studies with glucose and sucrose enhanced the 1,3-PDO production but the preferential order of co substrates was sucrose first, then glucose. The process yield obtained with sucrose-glycerol mixture was 98%, which is close to the theoretical yield of 100%. The work done provides the valuable information for further experiments with the isolated *K. pneumoniae* strain for enhancing 1,3-PDO production.

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